## TRANSLATOR'S CERTIFICATE

I, Elgin E. Marko, do hereby certify that I am fluent in the German and English languages. I prepared the translation into English of German Patent Application DE10033219(A1). It is true and accurate to the best of my ability.

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WO 99 17 798 A 1
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**The following statements are obtained from the documents filed by the applicant.**
An application for verification pursuant to § 44 PatG has been filed.
Neuroprotective effect of granulocyte colony stimulating factor (G – CSF)

The use of G – CSF (granulocyte colony stimulating factor) for the production of pharmaceutical preparations with a neuroprotective effect for the treatment of acute ischemic diseases such as apoplexia and neurodegenerative illnesses such as Parkinson's or Alzheimer's diseases.

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Description

[0001] Use of G – CSF (granulocyte colony stimulating factor) for the production of pharmaceutical preparations with a neuroprotective effect for the treatment of acute ischemic diseases.

[0002] Stroke (apoplexia cerebri) is the third most frequent cause of death in the western industrialized nations, is one of the leading causes of long-term disability and care requirements and is therefore, from an economic perspective, in the single most costly group of illnesses. At the present time, approx. 150,000 persons annually suffer a stroke within Germany. Of these, 15 – 20 percent of patients die within the first four weeks. Only approximately one-third of the surviving patients recover without major lasting disabilities, while another third remains affected by lasting severe disabilities due to paralysis or other neurological failures. In 80 percent of the patients, the stroke is due to a circulation disturbance with subsequent ischemia within a circumscribed region of the brain. Circulation disturbances in the brain usually occur either in a macro-angiopathic manner due to thromboembolism or hemodynamic reductions of flow speeds, or in a micro-angiopathic manner due to blood pressure related arteriosclerosis of the small intracerebral end arteries. As such, a series of risk factors favor the occurrence of a stroke. In particular, these include arterial hypertension; numerous heart disorders which are connected with a heightened risk of embolism – especially atrial fibrillation; diabetes mellitus, smoking cigarettes, blood coagulation disturbances and – to a lesser degree – hypercholesteraemia. Embolic or local thrombotic occlusion of one of the large brain supplying arteries leads to territorial infarctions, that is, infarctions which affect a circumscribed region within the supply region of a specific brain artery. This most frequently affects the supply region of the arteria cerebri media, producing a media territorial infarction with a corresponding "media syndrome". This is also the single most frequent manifestation of a stroke. Up to now, thromboembolytic therapy only promises success in selected patients. In recent years, new pathophysiological knowledge and methods have brought about significant changes in the diagnostics and therapy of acute cerebral ischemia. Thrombolysis offers the option of therapeutic intervention within a "therapeutic window" of 3 to 6 hours after the infarction occurs. Its goal is the rapid dissolution of the vascular occlusion and therewith, the restoration of cerebral circulation and improvement of the neurological symptoms. This is based on the pathophysiological idea that the re-opening of an occluded cerebral blood vessel supports the maintenance of hypo-perfused, reversibly damaged grain tissues (the so-called ischemic penumbra) and therewith, the restoration of neuronal functions. However, up to now, this treatment can only be carried out in certified neurological centers. Furthermore, the authorization to market rt-PA for strokes within Germany is still pending. Lysis therapy after 6 hours is regarded as being particularly high in side effects (increased number of intracranial bleeding occurrences) and should therefore not be performed. Other therapies have not been evaluated at this time. Here, we primarily refer to so-called growth factors (bFGF)

and pharmaceutical products which block thrombocyte adhesion (anti-GP IIb / IIa, Abcizimab). In recent years, numerous neuroprotective substances were examined in clinical studies. Unfortunately, none of the substances tested so far, which all show a

neuroprotective effect in animal models, were able to prove their benefits in clinical practice. In particular, the glutamate antagonists, free radical catchers and NMDA antagonists did not result in clinical benefits, or even showed significant side effects which make clinical use impossible (e.g. psychosis, etc.). Other substances, which inhibit leukocytary wall adhesion (anti-ICAM-1) or the inhibitor of glutamate transmitted NO synthetase (Lubeluzol) did not result in positive effects.

[0003] The task of the invention is to ease or remove the consequences of acute ischemia by administering substances with neuroprotective effects. This task is solved by the procedure with the attributes of claim 1.

[0004] The use of G-CSF (granulocyte colony stimulating factor) for the production of pharmaceutical preparations with a neuroprotective effect represents a successful therapeutic approach through the use of neuroprotective growth factors.

[0005] G-CSF, which is the English abbreviation for "Granulocyte Colony Stimulating Factor", is an endogenic and haematopoietic growth factor which regulates the maturing processes, proliferation and differentiation of neutrophile granulocytes. G-CSF is produced naturally through various monocytes, macrophages and T lymphocytes as a glycoprotein and is one of the cytokines. G-CSF is already in use as a recombinant human factor Filgrastim (Neupogen ® / company Amgen GmbH, CAS No. 121181 – 53 – 1) in the treatment of neutropoenia and neutropoenic fever. Other recombinant human G-CSF includes Lenograstim and Molgramostim. A neuroprotective effect of G-CSF has not been described yet.

[0006] A 90 minute ischemia was induced in 24 Wistar rats by means of the internationally recognized thread model as per Longa, et al. 30 minutes after the ischemia had been induced, 12 rats (n = 12) received infusions of 2 ml NaCl intravenously over a total of 90 minutes; these rats served as the control group (C). The therapy group (T; n = 12) received 20 micrograms G-CSF throughout the same time period, dissolved in 2 ml NaCl. Prior to the induction of the ischemia and 1, 2, 3, 4 and 24 hours later, ELISA (Biosource Europe, Fleurus, Belgium) was used to determine the concentration of Interleukin 1-beta, IL-2, IL-6 and IL-10. After 24 hours, the brains were removed and 5 brain sections were taken from the frontal region, with a thickness of 2 mm. TTC tinting was used to determine the infarction and brain oedema size from sections 1, 2, 4 and 5. Section 3 received further histological processing. In order to prove the cerebral invasion by neutrophile granulocytes, a myeloperoxidase tint MPO (DAKO, Carpinteria, CA, USA) was done. Through the addition of an anti-G-CSF antibody, evidence of the presence of the G-CSF receptor, not yet described, was confirmed.

[0007] Cerebellar granulocytes were gained from P7 mice and processed and cultured in accordance with an established cell culture model. After 7 days, the granula cells were treated with G-CSF, and glutamate was added after 30 minutes. In order to test the

survival rate of the cells,.3-(4,5 dimethyl thiazole 2-yl) 2,5 diphenyl tetrazolium bromide (MTT, Sigma, Munich) was applied 2 hours after the glutamate stimulation. After another 4 hours, the cells were lysed with 1 % SDS. The samples were measured at 590 nm optic density. Furthermore, the G-CSF receptor was confirmed by means of PCR. For this purpose, RNA was extracted from mouse brains (RNA kit, AGS, Heidelberg). 10 micrograms of RNA were transcribed with MMLV reverse transcriptase. For the PCR,

primers of Exon 5 and 7 of the G-CSF receptor were utilized (Ashihara, 1997). The statistical analysis was carried out for the animal experiment as per Anova with Bonferroni correction for multiple groups.

[0008] The control group and the therapy group were not different in terms of the measured physiological parameters (BGA, HCT, blood pressure and body weight). After 24 hours, a slight increase in the neutrophile granulocytes in the blood was determined. This was not significant. The infarction size in the TTC sections was 6.7 % + / - 6.7 % (n = 12) of the total brain volume for the G-CSF treated group (T) and was thereby significantly ($p < 0.05$) smaller than that of the control group with 22.7 % + / - 6.3 % (n = 12). The calculated brain oedema was also significantly lower in the G-CSF group with 4.7 % + / - 6.6 % ($p < 0.05$) than in the non-treated group with 12.0 % + / - 6.1 %. For all measured interleukins, except for IL-2, significantly lower serum parameters were proven in the G-CSF group ($p < 0.05$). In the histological evaluation of brain section 3, only an increase in the invasion of neutrophile granulocytes was found in the MPO tint for both animal groups. This increased with the size of the infarction. Significant differences were not observed. In the brain sections, the bonding of anti-receptor G-CSF was shown both to neurons and to axons and dendrites. In the cell culture, a significant reduction of cell death was observed. This effect increased with higher dosages of G-CSF. The PCR proved the mouse receptor in the brain tissues by means of PT-PCR. The PCR product had the expected size of 567 bp and was verified by means of PCR sequencing.

[0009] The results show that G-CSF has neuroprotective attributes. These were demonstrated both in animal experiments via a reduction of the infarction area and brain oedema in the G-CSF treated group and also in cell cultures through G-CSF-reduced glutamate damage. Acting mechanisms consist of an activation of the intracerebral G-CSF receptor and a reduction of specific interleukins which intervene in the inflammatory processes in cerebral ischaemia. Since G-CSF is produced endogenically, has a cerebral receptor and possesses neuroprotective attributes, it is one of the series of neutrophiles such as BDNF, IGF and NGF. Side effects were not found in our experiment.

[0010] Since G-CSF is a medication with a low rate of side effects, which has been established for several years, our possible application for cerebral ischemia is of great practical relevance. G-CSF represents an effective pharmaceutical active substance for thus far unsatisfactory therapy for strokes.

Patent claims

1. Use of G-CSF (granulocyte colony stimulating factor) for the production of pharmaceutical preparations with a neuroprotective effect for the treatment of acute ischaemia and neurodegenerative diseases.

2. Use in accordance with claim 1, wherein G-CSF is a polypeptide and/or a glycoprotein, and/or a derivate or analogue of G-CSF, which possesses granulocyte cell colony stimulating activity.
3. Use in accordance with claims 1 and 2 wherein G-CSF is produced either chemically – synthetically (such as derivates, analogues, isomers) and/or

with recombinant methods and/or is isolated from G-CSF forming cells.
4. Use in accordance with claim 1 wherein acute ischemia, e.g. of the types occurring in apoplexia, skull – brain trauma or tumours, is treated.
5. Use in accordance with claim 1, wherein neurodegenerative diseases such as Parkinson's and Alzheimer's are treated.
6. Use in accordance with claim 1, wherein the pharmaceutical preparation can be solid, liquid or in the form of an aerosol (such as a spray).
7. Commercial package containing a G-CSF containing pharmaceutical preparation together with instructions for the use of G-CSF in the neuroprotective treatment of acute ischaemia or neurodegenerative diseases.